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Effects of probe–amphiphile interaction on pyranine proton transfer reactions in lecithin vesicles

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Abstract

Photoacids and photobases are powerful tools for the discrimination, in real time, between the preferential pathways in H^+/OH^- transfer processes in biological and biomimetic systems which can determine the origins of crucial biochemical processes. In this study, the binding of 8-hydroxy-1,3,6-pyrenetrisulphonate sodium salt (pyranine) to phosphatidylcholine (lecithin) vesicles was investigated (by time-resolved and steady state fluorometry and laser-induced pH jump measurements) as a function of the probe and electrolyte concentration to determine how these parameters affect the properties of the photoacid. The ratio (R) between the fluorescence intensities at 510 nm (dissociated form) and 440 nm (undissociated form) can be related to the excited state degree of dissociation (D (%)). The binding of the probe to the internal leaflet of lecithin small unilamellar vesicles (SUVs) is larger than that to the external leaflet. The addition of salt up to 2 M does not prevent binding even at low probe concentrations. Time-resolved emission decay measurements also show the probe–lipid interaction. The lifetime of the undissociated form is equivalent to that in alcoholic solutions (approximately 3.8 ns). The pyranine ground state re-protonation rate constant (k_{obs}) is dependent on the probe content per vesicle. With 550 pyranine molecules per vesicle, k_{obs} is constant within a large range of salt concentration. In this situation, an alternative route is proposed (compared with direct PO^-/H^+ recombination), in which proton transfer between neighbouring species maintains an unchanging recombination rate. The data presented show that careful analysis of pyranine H^+ transfer data is necessary for biomimetic and biological or reconstituted systems. © 1997 Elsevier Science S.A.

Keywords: Binding; Lecithin vesicles; pH jump; Photoacids; Pyranine

1. Introduction

Bioenergetic processes usually involve proton gradients across membranes. Protons are actively transported by phosphorylating membranes in mitochondria and chloroplasts, and the resulting electrochemical gradient can be coupled to the synthesis of adenosine triphosphate (ATP) [1,2]. The study of the formation and relaxation of proton gradients is therefore very important for understanding "in vivo" energy conversion.

Photoacids and photobases are useful compounds which permit H^+ and OH^- diffusive motions to be monitored in real time (nanoseconds to microseconds) [3–5]. In particular, pyranine, which has a very high excited state H^+ dissociation rate constant in bulk water compared with its singlet excited state lifetime [6], has been extensively used as a proton emitter in laser-induced pH jumps [7–10]. Typically, a short, intense laser pulse produces, within a few nanoseconds, the discharge of protons in solution lowering the local pH by 3–4 units. Using this technique, as well as fluorescence spectroscopy, pyranine and other aromatic alcohols have been employed either as proton emitters or pH probes in biomimetic systems, such as micelles [11–16], small unilamellar vesicles (SUVs) [16–19] and multilamellar vesicles [19–21]. Given the triply negative charge of undissociated pyranine, it has been assumed that the dye partitions preferentially in the water phase in anionic and neutral micelles or vesicles [11–14,18–20,22]. For example, Clement and Gould [19], although commenting that DMPC membranes could bind somewhat higher levels of pyranine, did not include interfacial effects in their analysis. Kotlyar et al. [17] incorporated high pyranine concentrations (approximately 10 mM) in submitochondrial particles but did not consider the effects due to H^+ interchange between bound pyranines or other interfacial effects. Kamp et al. [22] employed pyranine as an internal "pH meter" in lecithin vesicles. By assuming that the dye resides exclusively in the aqueous

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internal compartment of lecithin vesicles, and by monitoring the dye fluorescence response with imposed pH gradients, these workers obtained the internal pH.

Two serious objections to this method are as follows. First, the pH measured by the fluorescence technique requires a knowledge of the pK_a value of the entrapped probe. If the probe resides exclusively in the aqueous phase, its pK_a value will be the same as for bulk aqueous solutions, with the appropriate corrections for salt effects on the pK_a value. However, if the probe partitions between the aqueous and membrane (bilayer) pseudophases, the experimental pK_a value will reflect the acidity constants of the bound and unbound species, the distribution coefficients of the species and the relative activities of H^+ in the internal aqueous compartment and bound or close to the bilayer [23]. Second, considering the usual concentration of entrapped probe (approximately 5×10^{-4} M [22]), the binding of pyranine could affect the local dipolar potential of the bilayer. Binding of a triply negative charged probe to lecithin will result in a local excess of negative charge compromising the neutral nature of the interface. This local inhomogeneity could facilitate proton movements in or through the bilayer and even flip-flop movements of species added externally to the vesicle preparation.

We have shown previously that pyranine, despite its polyanionic character, binds significantly to zwitterionic micelles of lysolecithin, sulphobetaines and lecithin vesicles [24,25]. However, the data on lecithin vesicles were not sufficiently detailed and require further investigation. In this study, the effect of salts on the binding properties of pyranine to the internal and external leaflets of lecithin vesicles is investigated. It is found that salt addition does not preclude binding even with a concentration of NaCl as high as 2 M. Furthermore, it is shown that the pH jump re-protonation rates of encapsulated pyranine depend dramatically on the entrapped probe concentration.

2. Experimental details

Egg yolk phosphatidylcholine (lecithin) was purified by the method of Hanahan et al. [26]. Pyranine (Eastman Kodak) was recrystallized three times from acetone–water (90 : 10, v/v) and showed no impurities in thin layer chromatography [27].

SUVs were prepared as follows. A film of lecithin was prepared by evaporation of the solvent from a solution of phosphatidylcholine (PC) in $CHCl_3$ with an N_2 flow. After drying the film under vacuum for 2 h, the lipid film was hydrated with an appropriate concentration of NaCl stock solution containing pyranine. The lipid dispersion (2 ml) was vortexed and sonicated (Braunsonic 1510 Melsungen AG) in an ice bath for ten repeated cycles of 1 min sonication and 1 min interval. The suspension was then ultracentrifuged at 18 000 rev min^{-1} for 1 h (Hitachi, model Hinac CR-20B2) to remove titanium dust and large aggregate particles. Non-entrapped pyranine was separated from pyranine-containing vesicles by gel chromatography of 2 ml samples in Sephadex G-25 columns (2 cm×25 cm) which had previously been saturated with lecithin, equilibrated and eluted with isotonic NaCl solution. Pyranine-containing vesicles were collected in the column void volume (V_0). The amount of residual pyranine bound externally to the vesicles could be assessed by monitoring the emission quenching of the probe fluorescence by added chloroquine (ClQ) before and after vesicle dissolution with Triton X-100 [28]. In this methodology, advantage is taken of the very low permeability of ClQ through the lipid bilayer. At pH ~ 5, ClQ is doubly charged and therefore unable to permeate into the vesicle internal compartment [29–31]. The extent of quenching before bilayer solubilization was lower than approximately 10% confirming the entrapment of pyranine. External pyranine arises from either sorption of the probe externally to the vesicles or vesicle rupture during storage and manipulation. However, the contribution of external probe is almost within experimental error (about 5%–10%), and its contribution is neglected hereafter. A detailed description of the ClQ/pyranine assay will be presented separately [28].

Large unilamellar vesicles (LUVs) were prepared as follows. An NaCl–pyranine aqueous solution and ethyl ether were added to a lecithin film and sonicated for 2 min in an ice bath, followed by rota-evaporation (reverse phase method) [32]. External pyranine and small vesicles were removed as above by eluting the sample in a Sephadex G-25 column (2 cm×25 cm). For all vesicle preparations, the phospholipid concentration was determined by the method of Rouser et al. [33].

Laser flash photolysis (Applied Photophysics) measurements were carried out using the third harmonic of an Nd–YAG laser ($\lambda = 355$ nm). The re-protonation kinetics of pyranine were monitored (pulsed 150 W xenon lamp) at 455 nm (the absorbance wavelength maximum of dissociated pyranine). Transient absorbance changes were captured in a digital oscilloscope (HP-54510C) and transferred to a 386-PC-compatible microcomputer for data analysis. The values of the observed re-protonation rate constants (k_{obs}) are the average of at least ten accumulated transients.

Fluorescence measurements were performed in a SPEX DM 3000F fluorometer. Emission and excitation spectra were computer corrected (SPEX software). The band overlap due to overlap of the emission of the undissociated species with that of the dissociated species was corrected using the following expression [3]

$$I_{(510)corr} = I_{(510)meas} - (0.318 \times I_{(440)meas}) \quad (1)$$

where $I_{(510)corr}$ is the true fluorescence emission intensity at 510 nm and $I_{(510)meas}$ and $I_{(440)meas}$ are the measured fluorescence emission intensities due to the dissociated (PO^-*) and undissociated (POH*) forms of pyranine respectively. The factor $(0.318 \times I_{(440)meas})$ is the contribution of the 440 nm emission (POH*) at 510 nm. The contribution of the 510

nm emission (PO^-*) at 440 nm is practically zero and therefore $I_{(440)corr} = I_{(440)meas}$.

Time-resolved fluorescence measurements were performed in an LS100 fluorometer (PTI, Canada) using the 337 nm line of N_2 for the excitation system. The lamp profile observed with empty vesicles had a full width at half-maximum (FWHM) of approximately 2 ns. The emission lifetimes were obtained with the deconvolution software provided by PTI (Canada). The quality of the vesicle preparations was determined by measuring their hydrodynamic radius (R_h), weight-averaged molecular mass ($\bar{M}_w$) and radius of gyration (R_g). These experiments were performed using a quasi-elastic laser scattering (QELS) system (for instrumental details, see Ref. [24]) for the determination of R_h and a DAWN multi-angle laser scattering (Wyatt Technology) apparatus for $\bar{M}_w$ and R_g. A brief outline of these results, adequate for the present purposes, is given in this paper; a full report will be presented elsewhere. For the SUV preparations, the above parameters show a slight dependence on the salt concentration and dilution; for the selected experimental conditions (see footnote of Table 2), the mean values are as follows: $\bar{M}_w = 1.89 \times 10^7$ g mol^{-1}, $R_g = 48$ nm and $R_h = 37$ nm. For LUV size determination by QELS, we obtained $R_h = 115$ nm. These values are in good agreement with the literature [32], demonstrating that the encapsulation of pyranine does not change the size of the vesicles significantly.

3. Results and discussion

The extent of excited state prototropism of pyranine to the solvent (water and several other media) can be determined by following the emission of the excited state conjugate acid/base species in a solution containing the undissociated species [34,35]. In bulk water, with a pH between the ground and excited pK_a values, the emission arises mostly from the prototropically formed excited state base. On decreasing the solution pH, excited state re-protonation competes with fluorescence decay and the emission of the undissociated species begins to increase. Fig. 1 depicts this behaviour showing the emission profiles of pyranine as a function of pH. Clearly, a decrease in pH is accompanied by an increase in the emission band centred at 440 nm (excited acid) and a decrease in that at 510 nm (excited base). Alternatively, the extent of dissociation can be modified by decreasing the amount of water (addition of alcohol, for instance), which hinders the excited state prototropism and results in an increase in undissociated pyranine (POH*) emission [35].

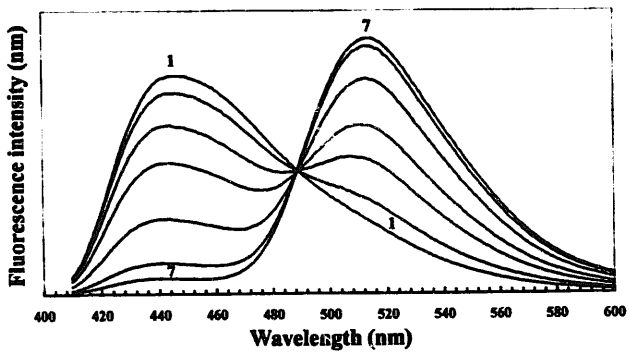


Fig. 1. Pyranine corrected fluorescence emission spectra as a function of H^+ concentration. $HClO_4$: 1, 3 M; 2, 2 M; 3, 1 M; 4, 0.5 M; 5, 0.1 M; 6, 0.01 M; 7, 0.001 M; [pyranine] = 5×10^{-6} M; $\lambda_{exc} = 403$ nm; excitation and emission slits fixed at 0.5 mm; room temperature (approximately 23 °C).

A useful parameter used to express the extent of excited state dissociation is the 510 nm/440 nm fluorescence emission intensity ratio (R) [25]. This ratio is preferred to the intensities, since changes in the emission under investigation are usually small. The relationship between R and the extent of excited state prototropism is as follows. The fluorescence quantum yields of POH* and PO^-* are almost unity [6] and, in the presence of the prototropic reaction, Eq. (2) holds for pyranine [35,36]

$$\frac{\phi_{POH}}{\phi^0_{POH}} + \frac{\phi_{PO^-}}{\phi^0_{POH}} = 1 \tag{2}$$

where ϕ^0_{POH}, $\phi^0_{PO^-}$, ϕ_{POH} and ϕ_{PO^-} are the fluorescence quantum yields of POH and PO^- in the absence and presence of the excited state prototropic reaction respectively. In the condition in which POH is the main species in the ground state, Eq. (2) can be approximated to

$$\frac{\phi_{POH}}{\phi^0_{POH}} + \frac{\phi_{PO^-}}{\phi^0_{POH}} = 1 \tag{3}$$

Under these conditions, POH deactivates almost exclusively by fluorescence emission either by the blue (POH*) or the green (PO^-*) species and the excited state reactions are adiabatic.

Letting

$$\frac{\phi_{PO^-}}{\phi_{POH}} \approx \frac{I_{(510)corr}}{I_{(440)corr}} = R \tag{4}$$

the extent of dissociation (D (%)) can be defined as

$$D\,(\%) = \frac{I_{(510)corr}}{I^0_{(440)corr}} \tag{5}$$

Combining Eqs. (3)–(5), the relationship between D (%) and R is obtained

$$D\,(\%) = \frac{R}{1+R} \tag{6}$$

Another advantage of employing R is that we do not need to know the probe concentration. For example, partitioning of the dye at the interface of an aqueous zwitterionic micelle is accompanied by a decrease in R (increase in the 440 nm emission), i.e. reduced photodissociation [25,37]. This effect is analogous to an increase in salt or organic solvent concentration (ethanol, for example) in bulk water solution and reflects the diminished capacity of the photodissociation of pyranine when the water concentration is reduced. The association of R values with the probe location or the local

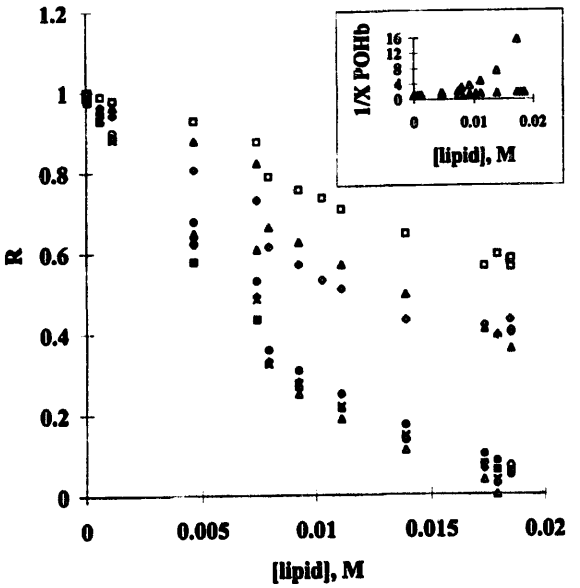


Fig. 2. Normalized fluorescence intensity ratio (R) ([pyranine] = 5×10^{-6} M) vs. lipid concentration at different ionic forces. NaCl: ■, 0 mM; ○, 5 mM; ◆, 10 mM; ×, 20 mM; ●, 50 mM; △, 100 mM; ◇, 200 mM; ▲, 500 mM; □, 1000 mM. Inset: $1/X_{POH_f}$ vs. lipid concentration: △, 10 mM of NaCl; ▲, 1000 mM of NaCl.

concentration of water is, however, unfeasible since the state of the dipolar region of the bilayer, where the probe is probably located, in terms of the ionic strength, polarity and other parameters, cannot be described completely. The choice of R as the analytical parameter stems from the small changes in the intensity of the fluorescence maximum or the emission quantum yields of POH* and PO^-* on binding to lecithin and the very low absorbance of the entrapped probe over the light scattering profile of the vesicles. Due to these experimental difficulties, which do not allow the concentration of the entrapped probe to be determined precisely, R is a suitable parameter. Changes in the R value on binding, although containing the approximations expressed in Eqs. (3) and (4) and the unknown quantum yield of the bound probe, reflect an association without requiring the probe location or the local state of the solvent to be established exactly.

Initially, to obtain a rough estimate of POH–lecithin affinity, R was determined for a large series of samples with different salt and lipid concentrations. In these experiments, the dye was added externally to the vesicles, i.e. the experimental difficulties of the incorporation and removal of the non-encapsulated dye applied to all of the selected conditions. Plots of the normalized R values against the lipid (vesicle) concentration for a series of electrolyte concentrations are presented in Fig. 2. A decrease in R is observed with increasing lipid concentration showing the reduction in D (%) with binding. An increase in salt concentration is accompanied by a decrease in externally bound probe (higher R). It should be noted that, even with 2 M salt, the complete recovery of R towards the values observed in bulk solution is not accomplished, showing clearly the existence of significant non-coulombic terms in the binding of pyranine.

The association constant (K) of pyranine to the external vesicle compartment can be estimated on the basis of Eq. (7)

$$POH_f + \text{lipid} \overset{K}{\rightleftharpoons} POH_b \tag{7}$$

$$K = \frac{[POH_b]}{[POH_f] \times [\text{lipid}]} \tag{8}$$

where [lipid], $[POH_b]$ and $[POH_f]$ are the equilibrium concentrations of the probe-free lipid and the bound and free probes respectively. Using this simple approach and considering the relevant lipid concentration of half of the titrated amount (external vesicle bilayer leaflet), the condition of [lipid] $\gg$ $[POH_b]$ is fulfilled in most of the experiments. Within these constraints, it is straightforward to simplify Eq. (8) to

$$K \times [\text{lipid}] + 1 = \frac{[POH]_{total}}{[POH_f]} \tag{9}$$

where

$$\frac{[POH]_{total}}{[POH_f]} = \frac{1}{X_{POH_f}}$$

Accordingly, the binding constant of pyranine to the external vesicle compartment can be obtained from the slope of a plot of $1/X_{POH_b}$ against [lipid]. In the inset of Fig. 2, $1/X_{POH_b}$ is plotted for two electrolyte concentrations (0.01 M and 1 M salt); clearly K is lowered with increasing salt concentration. The fitting to Eq. (9) is reasonable for 1 M, whereas for 0.01 M a pronounced curvature is observed. In general, the fitting is better at higher salt concentrations. Given the phenomenological nature of Eq. (7), its stoichiometry was not varied to improve the fitting. Values of K were simply calculated from the best regression lines from the plots given in the inset of Fig. 2. The K values for the investigated range of salt concentrations are presented in Table 1. The variations in K from 0 to 2 M salt are in reasonable agreement with a simple Debye–Hückel electrolyte screening effect on the partitioning equilibrium. For instance, by plotting the decadic logarithm of K vs. the NaCl activity (molal activity coefficients taken from Ref. [38]), a fair linear dependence is obtained with a slope of approximately unity. It is important to add once again that, even with a fairly high salt concentration, binding is still appreciable, showing the existence of non-coulombic terms in POH binding to lecithin. This effect is assigned to the specific strong inter-

Table 1
Binding constants (K) of pyranine to lecithin vesicles as a function of NaCl concentration

[NaCl] (M)	0	0.005	0.01	0.02	0.05	0.1	0.2	0.5	1.0	1.5	2.0
K (M^{-1})	2654	1415	969	809	634	733	161	168	84	55	30

Table 2
Ratio of the fluorescence intensities (R) between dissociated (PO^-) and undissociated (POH) forms of pyranine as a function of the salt concentration for the probe in bulk solution (conditions A*, B* and C* (R_{bulk})) and in lecithin vesicles (entrapped probe in SUV (conditions A, B and C); external probe with SUV (conditions D and E); entrapped probe in LUV (condition F) ($R_{vesicle}$))[a]

[NaCl] (mM)	R_{bulk}			$R_{vesicle}$					
	A*	B*	C*	A	B	C	D	E	F
12.5	21.4	16.2	18.8	3.8	1.5	1.3	17.1	14.8	5.6
20.0	21.3	19.9		2.7	1.9				5.0
200	26.0	25.0		4.7	3.8	2.8			
500						2.4			
1000	27.9	26.1		4.6	3.7	2.8			
2000	22.4	16.7	18.7	3.3	2.1		15.9	13.3	

[a]Conditions: R_{bulk}: eluting solutions used in size exclusion chromatography for vesicle preparations in conditions A*, B* and C*; $R_{vesicle}$: SUV with entrapped probe containing 550 (A), 280 (B) and 3 (C) pyranine molecules per vesicle; SUV with external (only) probe, [pyranine] = 1×10^{-5} M, [lipid] = 0.1 mg ml^{-1} (D) and [lipid] = 3 mg ml^{-1} (E); LUV with entrapped probe containing 380 pyranine molecules per vesicle (F).

action between ammonium and sulphonate groups, as previously observed with cetyltrimethylammonium (CTA) monomers or CTACl micelles and pyranine [35].

The binding of POH to the inner monolayer of lecithin SUVs was investigated using three distinct pyranine to vesicle ratios. Results with approximately 550, 280 and 3 probe molecules per vesicle are presented in Table 2 (A, B and C respectively). These ratios were obtained using the experimental radius of gyration ($R_g \sim 48$ nm, see Section 2) to calculate the internal vesicle volume and the pyranine concentration added to the sample preparation, i.e. 2, 1 and 0.01 mM for 550, 280 and 3 probe molecules per vesicle respectively. Comparing the R values for the POH prototropic process in bulk water at the same salt concentrations with those of POH included in the vesicles, lower R values were found for the latter. For bulk solution, $R \sim 22$ (i.e. $D \sim 96\%$, Eq. (6)), with an estimated error of 10%–15%, whereas for lecithin vesicle preparations A, B and C, $R \sim 3$ ($D \sim 75\%$). It should be noted that, up to 2 M NaCl, the decrease in water activity does not alter the extent of H^+ dissociation in the bulk [39,40]. The effect is therefore a direct manifestation of an excess POH* concentration (hindered excited state dissociation), which results in a decrease in R when the probe is in a region of lower water concentration.

In a previous study, pyranine has been shown to interact strongly with the ammonium moiety of lyso-PC micelles, i.e. with the positive end of the interfacial dipole [25]. Once in this situation, the probe is still relatively close to the bulk solvent, and excited state dissociation, although somewhat reduced, still occurs. This is not the case with cationic CTACl micelles or zwitterionic interfaces (sulphobetaine micelles, for example) which have the dipole in an opposite disposition compared with lyso-PC interfaces [24,25]. In these aggregates, probe–amphiphile interaction results in a deeper penetration of the probe in the lipid region of the macromolecular aggregate and therefore in an almost complete disappearance of pyranine photoacid properties. The relevance of this point is to stress that, even in the case of complete POH association with lecithin vesicles, H^+ transfer to the solvent, although diminished, occurs, i.e. binding of POH does not hinder excited state dissociation completely. In other words, the decrease in the R value from approximately 22 (bulk water, $D \sim 96\%$) to approximately 3 (interfacial POH, $D \sim 75\%$) on extensive binding of POH (see, for example, Table 2 (condition B) at 20 mM of salt) shows that, at the vesicle interface, dissociation is estimated to be hindered only by about 20%.

The data presented in Table 2 show that encapsulated pyranine has $R \sim 3$ irrespective of the amount of electrolyte added. Decreasing the amount of pyranine per vesicle from 550 to 280 and finally to 3 leads to a slight decrease in the mean value of R from 3.8 to 2.6 to 2.3 (conditions A, B and C respectively). By considering that the pyranine–vesicle affinity for the internal surface is the same as that for the external surface, the reduction in K from 969 to 30 (from 0.0125 M to 2 M, Table 1) will not result in probe dissociation. Higher values of R with higher pyranine load can therefore be interpreted by packing constraints. With 550 POH molecules per vesicle, the internal surface can barely accommodate such a load. In the case of LUVs, condition F (approximately 380 pyranine molecules per vesicle) in Table 2, the R values are higher than those for SUVs. For LUVs, the internal vesicle volume is increased by about 14-fold compared with SUVs. For comparison, the R values for external POH at fixed dye concentration (1×10^{-5} M) and varying lecithin concentration (0.1–3 mg ml^{-1}) are included in columns D and E respectively. It is observed that an increase in lecithin by 30-fold is accompanied by a decrease in R in agreement with Eq. (7). Given the fairly high pyranine–lecithin affinity, it becomes apparent that the clue to understanding the differences in R summarized in Table 2 stems from a consideration of the amount of the aqueous phase. For external POH, the volume of the aqueous phase is very large, and therefore the equilibrium presented in Eq. (7) is appropriate. However, for SUVs, the aqueous volume is low and should be explicitly included in the association equation. Furthermore, a comparison between SUVs with small probe to vesicle ratios (condition C, Table 2) and LUVs with high

Table 3
Comparison of POH* (445 nm) and PO^-* (510 nm) lifetimes (τ) between bulk and interfacial conditions for SUV with incorporated probe containing 550 pyranine molecules per vesicle (A) and 280 pyranine molecules per vesicle (B)

[NaCl] (mM)	Bulk		Lecithin vesicle			
	τ(510 nm) (ns)	τ(445 nm) (ns)	τ(510 nm) (ns)		τ(445 nm) (ns)	
	A	A	A	B	A	B
12.5	5.5	4.6				
200			5.3	5.4	3.7	4.0
1000			5.1	5.1	3.7	3.9
2000	5.4	4.7	5.2		3.8	

probe to vesicle ratios (condition F, Table 2) shows a larger R for LUVs, pointing to a distinct probe–lecithin affinity and water restrictions for SUVs.

Other evidence reflecting the binding of pyranine can be obtained by measuring its emission decay. In bulk water, POH* has a lifetime (τ) of the order of 5.0 ns, whereas in alcoholic solution, the decay is shortened to approximately 3.7 ns [37]. In Table 3, the measured τ values at the emission wavelengths of POH* and PO^-* in bulk solution and entrapped in lecithin vesicles are summarized. In bulk solution, from 12.5×10^{-3} M to 2 M NaCl, the τ values are in good agreement with previous determinations [33,35], i.e. τ(445 nm) ~4.6 ns and τ(510 nm) ~5.4 ns. With vesicles, the τ values at 445 nm are consistently shorter (approximately 3.8 ns), whereas those at 510 nm remain of the same order as for bulk solution. τ_{POH}* can therefore be related to a medium of lower polarity. The analysis of the POH* and PO^-* decays was restricted to a single exponential since χ^2 values were close to unity, and the assay objective was to obtain an overall view of the emission decay pattern without going into more detailed kinetic analysis.

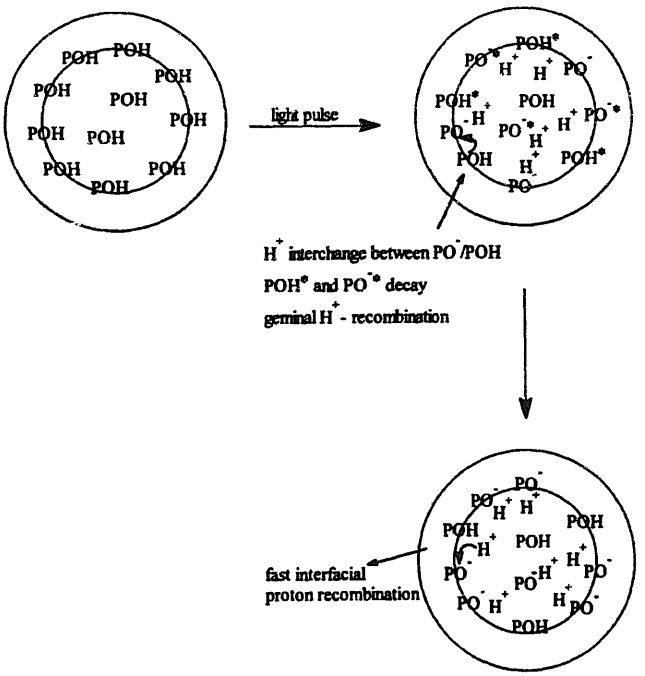
Effects on the H^+ transfer kinetics due to the concentration of the entrapped probe are revealed when the ground state re-protonation rate constants (k_{obs}) are measured. In Table 4, values of k_{obs} in bulk solution and in vesicles (same conditions as presented in Table 2, except for condition C whose transient signals were too weak to be measured) are summarized. The data are presented as observed first-order rate constants [6,8,35] to avoid any assumptions about the pH inside the vesicles. In bulk solution and in vesicles (conditions B, D, E and F), with the same salt concentration (i.e. the same row in Table 4), electrolyte effects on k_{obs} from 12.5 mM to 2 M NaCl follow the standard electrostatic screening effect (discrepancies between individual values are due to variations in the pH of each preparation) and are equivalent. For condition A, however, k_{obs} is constant and independent of the amount of added salt. Comparing columns A and B (lecithin vesicles) in Table 4, it is clear that k_{obs} remains of the order of 4.7×10^5 s^{-1} from 12.5 mM to 2.0 M NaCl in column A, whereas in column B, k_{obs} decreases from 3.6×10^5 s^{-1} to 0.6×10^5 s^{-1}. This effect shows that, with the larger POH load (condition A, 550 pyranine molecules per vesicle), an alternative pathway to direct PO^-/H^+ recombination appears.

For an analysis of this effect, it is necessary to consider the vesicle internal free volume for the H^+ transfer reaction. This volume can be estimated by subtracting from the total internal volume (R_g ~480 Å) the volume occupied by the encapsulated probe. The volume occupied by each probe is that of the Coulomb cage, which is defined as the size at which the electrostatic and thermal energies are equal [41]. Within the cage, recombination occurs in a few nanoseconds and is therefore outside the experimental time resolution. Pyranine in low salt aqueous solution presents a cage radius of approximately 30 Å [10,37]. Thus, taking these two radii, the free volume accounts for about 85% of the total vesicle volume (SUV volume $=4.63\times10^8$ $Å^3$; volume of spherical cage $\times 550 = 6.22\times10^7$ $Å^3$). With increasing salt concentration, the cage size is reduced to about 6 Å and therefore, in this condition, the free volume does not differ significantly from the total internal volume. Therefore it can be concluded that, although the encapsulated load is high, it does not

Table 4
Pyranine re-protonation rate constants (k_{obs}) as a function of added salt in bulk solution and in vesicles (for assay conditions see footnote in Table 2)

[NaCl] (mM)	k_{obs} ($\times10^5$ s^{-1}) (bulk)	k_{obs} ($\times10^5$ s^{-1}) (lecithin vesicle)			
	A, B, D and E[a]	A	B	D and E	F
12.5	4.69	4.77	3.62	5.85	3.67
200	4.00	3.55	0.94		
1000	2.99	4.34	0.75		
2000	0.91	4.73	0.64	0.62	

[a]Bulk k_{obs} values were obtained from at least three independent determinations; numbers presented are the arithmetic mean; standard deviation, ±10%.



Scheme 1. Alternative H^+ transfer route for vesicles with high load.

impose a drastic restriction on the reaction. Moreover, the binding of pyranine to the vesicle internal surface would decrease the total volume occupied by the probe and therefore relax the above size considerations further.

Given the lack of salt effects on k_{obs} in condition A (Table 4) and the fact that the photoacid properties of pyranine are only slightly reduced on binding to lecithin, an alternative re-protonation route is necessary. This route could arise from a combination of H^+ interchange between bound undissociated probe and neighbouring dissociated probe with fast interfacial H^+ diffusion. This is shown in Scheme 1. In Scheme 1, the first frame represents a vesicle with a large load of encapsulated probe partially bound to the internal surface. Following light excitation, a prototropic reaction occurs. Given the close proximity of H^+ emitters, it is highly probable that cage recombination will quickly repopulate a portion of the POH species. This situation is presented in the next frame where H^+, POH*, PO^-*, POH and PO^- coexist. An H^+ interchange reaction between POH and PO^- would maintain the population of bound PO^- approximately constant, with final reallocation of an H^+ species. The interchange step does not alter the transient signal since there is no net reduction in the solution absorbance at the selected wavelength. The binding of this high load of POH creates a negatively charged layer at the interface which will attract positively charged ions and therefore H^+. In order to maintain k_{obs} invariant, the recombination step occurring at the vesicle internal surface must be fast. A possibility within current concepts of proton lateral diffusion at the interface (see Ref. [41]) proposes an increase in H^+ diffusion of approximately 20-fold. When the encapsulation load is lowered to half or less, the average distance between pyranines decreases considerably and the prototropic cycle is normal, as demonstrated recently for H^+ recombination of PO^- bound to sulphobetaine micelles [24].

4. Conclusions

In this study, the association of pyranine with lecithin vesicles has been shown to be quite high even in the presence of concentrated electrolyte. Binding alters the probe photoacid properties slightly, but demonstrates the ability of a compound with three negative charges to reside in close proximity with the zwitterionic interface. When the amount of encapsulated probe is increased, interfacial reaction offers an alternative route to that of simple PO^-/H^+ transfer, which must be considered. The mechanism depicted in Scheme 1 is equivalent to a self-buffering effect which maintains invariant the rate of H^+ recombination at the interface.

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References

[1] P. Mitchell, *J. Theor. Biol., 62* (1976) 327–367.
[2] P. Mitchell, *FEBS. Lett., 222* (1987) 235–245.
[3] J.F. Ireland and P.A.H. Wyatt, *Adv. Phys. Org. Chem., 12* (1976) 131–221.
[4] S.G. Schulman, R.N. Kelly and N.J. Gonzalez, *Pure Appl. Chem., 59* (1987) 655–662.
[5] E. Pines and G.R. Fleming, *J. Phys. Chem., 95* (1991) 10 448–10 457.
[6] D. Huppert, M. Gutman and K.J. Kaufmann, *Adv. Chem. Phys., 47* (1981) 643–679.
[7] M. Gutman, *Methods Biochem. Anal., 30* (1984) 1–103.
[8] M. Gutman, *Methods Enzymol., 127* (1986) 522–538.
[9] M. Gutman and E. Nachliel, *Biochim. Biophys. Acta, 1015* (1990) 391–414.
[10] E. Pines and D. Huppert, *J. Phys. Chem., 87* (1983) 4471–4478.
[11] M. Gutman, E. Nachliel, E. Gershon and R. Giniger, *Eur. J. Biochem., 134* (1983) 63–69.
[12] P. Scherrer, U. Alexiev, T. Marti, H.G. Khorana and M.P. Heyn, *Biochemistry, 33* (1994) 13 684–13 692.
[13] U. Alexiev, T. Marti, M.P. Heyn, H.G. Khorana and P. Scherrer, *Biochemistry, 33* (1994) 13 693–13 699.
[14] M. Gutman, D. Huppert, E. Pines and E. Nachliel, *Biochim. Biophys. Acta, 642* (1981) 15–26.
[15] E. Nachliel and M. Gutman, *Eur. J. Biochem., 143* (1984) 83–88.
[16] E. Nachliel and M. Gutman, *J. Am. Chem. Soc., 110* (1988) 2629–2635.
[17] A.B. Kotlyar, N. Borokov, S. Kiryati, E. Nachliel and M. Gutman, *Biochemistry, 33* (1994) 873–879.
[18] M. Gutman, A.B. Kotlyar, N. Borokov and E. Nachliel, *Biochemistry, 32* (1993) 2942–2946.
[19] N.R. Clement and J.M. Gould, *Biochemistry, 20* (1981) 1534–1538.
[20] M. Gutman, E. Nachliel and S. Moshiach, *Biochemistry, 28* (1989) 2936–2940.

[21] M. Gutman and D. Huppert, *J. Biochem. Biophys. Methods, 1* (1979) 9–19.
[22] F. Kamp, D. Zakim, F. Zhang, N. Noy and J.A. Hamilton, *Biochemistry, 34* (1995) 11 928–11 937.
[23] C. Minero and E. Pelizzetti, *Adv. Colloid Interface Sci., 37* (1992) 319–334, and references cited therein.
[24] M.S. Baptista, I. Cuccovia, H. Chaimovich, M.J. Politi and W.F. Reed, *J. Phys. Chem., 96* (1992) 6442–6449.
[25] M.S. Baptista and M.J. Politi, *J. Phys. Chem., 95* (1991) 5936–5942.
[26] D.J. Hanahan, J.C. Dittmer and E. Warashina, *J. Biol. Chem., 228* (1957) 685–700.
[27] K. Kano and J.H. Fendler, *Biochim. Biophys. Acta, 509* (1978) 289–299.
[28] C. Fernandez and M.J. Politi, to be submitted.
[29] J.L. Irvin and E.M. Irvin, *J. Am. Chem. Soc., 69* (1947) 1091–1099.
[30] P. Nickel, *Pharm. Zeit., 113* (1968) 1609–1612.
[31] G.S.S. Ferreira, D.M. Périgo, M.J. Politi and S. Schreier, *J. Photochem. Photobiol. A: Chem.*, submitted for publication.
[32] F. Szoka, Jr. and D. Papahadjopoulos, *Annu. Rev. Biophys. Bioeng., 9* (1980) 467–508.
[33] G. Rouser, S. Fleischer and A. Yamamoto, *Lipids, 5* (1970) 494–496.
[34] E. Pines, D. Huppert and N. Agmon, *J. Phys. Chem., 95* (1991) 666–674.
[35] M.J. Politi and J.H. Fendler, *J. Am. Chem. Soc., 106* (2) (1984) 265–273.
[36] A. Weller, *Prog. React. Kinet., 1* (1969) 189–214.
[37] M.J. Politi, O. Brandt and J.H. Fendler, *J. Phys. Chem., 89* (1985) 2345–2354.
[38] R.A. Robinson and R.H. Stokes, *Electrolyte Solutions*, Butterworths, London, 1959.
[39] M.J. Politi and H. Chaimovich, *J. Solution Chem., 18* (1989) 1055–1067.
[40] D. Huppert, E. Kolodney, M. Gutman and E. Nachliel, *J. Am. Chem. Soc., 104* (1982) 6949–6953.
[41] M. Gutman and E. Nachliel, *Biochim. Biophys. Acta, 1231* (1995) 123–138, and references 66–69 cited therein.